

# T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR

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Regulatory T (Treg) cells safeguard against autoimmunity and immune pathology. Because determinants of the Treg cell fate are not completely understood, we have delineated signaling events that control the *de novo* expression of Foxp3 in naive peripheral CD4 T cells and in thymocytes. We report that premature termination of TCR signaling and inhibition of phosphatidylinositol 3-kinase (PI3K) p110 $\alpha$ , p110 $\delta$ , protein kinase B (Akt), or mammalian target of rapamycin (mTOR) conferred Foxp3 expression and Treg-like gene expression profiles. Conversely, continued TCR signaling and constitutive PI3K/Akt/mTOR activity antagonized Foxp3 induction. At the chromatin level, di- and trimethylation of lysine 4 of histone H3 (H3K4me2 and -3) near the Foxp3 transcription start site (TSS) and within the 5' untranslated region (UTR) preceded active Foxp3 expression and, like Foxp3 inducibility, was lost upon continued TCR stimulation. These data demonstrate that the PI3K/Akt/mTOR signaling network regulates Foxp3 expression.

Specialized cell types in multicellular organisms are defined by distinct patterns of gene expression (1). During their differentiation from hematopoietic stem cells, developing T cells undergo progressive restriction of their lineage potential. After the CD4/CD8 lineage choice in the thymus, CD4 lineage cells remain able to adopt a naive or regulatory cell fate, and naive CD4 T cells can opt for a range of Th lineages or, alternatively, become regulatory T (Treg) cells after activation (2, 3). The choice of Th lineage is important for effective immune responses to specific pathogens, and the balance between effector and regulatory cells is critical to ensure immune competence while avoiding immune pathology and autoimmunity. Thymus-derived Treg cells are generated via a TGF $\beta$  independent pathway that requires costimulatory signals (2–4) and typically express the signature transcription factor Foxp3, which confers regulatory T cell function (7–10). Differences between the TCR repertoires of conventional and regulatory CD4 T cells attest to the importance of MHC/peptide recognition and TCR signaling in conventional versus regulatory T cell differentiation (11, 12). Adaptive Treg cells can arise from naive peripheral CD4 T cells, for example by immunisation with low dose antigen and limited costimulation (13). TGF $\beta$  is a potent inducer of Foxp3 expression *in vitro* (14) and *in vivo* (15–17) and immunosuppressive drugs, such as rapamycin (18–20), act by as yet undefined mechanisms to induce Foxp3 expression (18) or to expand preexisting Treg cells (19, 20). To clarify the determinants of the Treg cell fate choice, we set out to identify signaling events that control Foxp3 expression. We show that activation of CD4 lineage thymocytes and peripheral T cells confers competence for the *de novo* expression of Foxp3 in a pathway that is independent of TGF $\beta$  and is instead controlled by phosphatidylinositol 3 kinase (PI3K), protein kinase B (Akt), and mammalian target of rapamycin (mTOR). The competence for Foxp3 induction is limited by TCR stimulation itself, and continued stimulation results in the loss of permissive chromatin modifications from the Foxp3 TSS and 5' UTR.

## Results

**Premature Withdrawal of TCR Signals and Inhibitors of the PI3K/mTOR Pathway Induce Foxp3 Expression in Activated CD4 T Cells.** Naive CD62L<sup>hi</sup>CD4<sup>+</sup>CD25<sup>−</sup> LN T cells were isolated by flow cytometry and labeled with CFSE. Residual Foxp3 expression was minimal as judged by intracellular staining (Fig. 1*a*, post sort) and remained unchanged after 18 h of activation with plate bound anti-TCR and anti-CD28 (Fig. 1*a*, 18h anti-TCR, anti CD28) and after another 36 h with anti-TCR (Fig. 1*a–d*, with TCR signaling). However, Foxp3 RNA and protein were markedly up-regulated when the same cells were activated for 18 h with plate bound anti-TCR and anti-CD28 and then maintained without TCR stimulation for 36 h (Fig. 1*a–d*, no TCR signaling). Hence, the continued availability of TCR signals appeared to control Foxp3 expression in newly activated CD4 T cells. TCR/CD28 engagement triggers multiple signaling pathways (21). To investigate which of these control Foxp3 expression, we screened small molecule inhibitors of enzymes involved in signal transduction. No increase in Foxp3 expression was seen when inhibitors of calcineurin/NFAT (cyclosporin A and FK-506), mitogen activated kinases (SB203580, PD98059), protein kinase-C (UCN-1028c, calphostin C, Myr-N-FARKGALRQ-NH2, Gö6976, Ro-32-0432, Ro-31-8220), glycogen synthase kinase-3 (SB21673), PPAR $\delta$  (GW501516), and  $\gamma$ -secretase/Notch (L-685458; data not shown) were added to 18 h activated CD4 T cells. By contrast, the PI3K inhibitor LY294002 potently induced Foxp3 in this assay (Fig. 1*a–c*, LY). Rapamycin, an inhibitor of the protein kinase mTOR, which lies in the same signaling pathway (25), also induced Foxp3 (Fig. 1*a–c*, rapa). The combination of LY294002 and rapamycin induced Foxp3 in  $\approx$ 75% of CD4 T cells (Fig. 1*a–c*, rapa+LY) and synergized with TGF $\beta$ , resulting in >90% Foxp3 induction in the absence of exogenous cytokines (Fig. 1*a–c*, TGF $\beta$ +rapa+LY). CFSE labeling ruled out the selective expansion of preexisting Foxp3<sup>+</sup> cells (Fig. 1*a*), and cell counts showed a substantial net increase in Foxp3<sup>+</sup> cell numbers (Fig. 1*c*).

*De novo* induction of Foxp3 by PI3K and mTOR inhibitors was formally demonstrated by using AND TCR transgenic *Rag1*<sup>−/−</sup> CD62L<sup>hi</sup>CD4<sup>+</sup>CD25<sup>−</sup> LN T cells, which are devoid of preexisting Foxp3<sup>+</sup> cells [Fig. 1*d* and [supporting information \(SI\) Fig. S1\*a\*](#)].

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The authors declare no conflict of interest.

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Data deposition: Gene expression data have been deposited in the ArrayExpress database, [www.ebi.ac.uk/ArrayExpress](http://www.ebi.ac.uk/ArrayExpress) (accession no. E-MIMR-1241).

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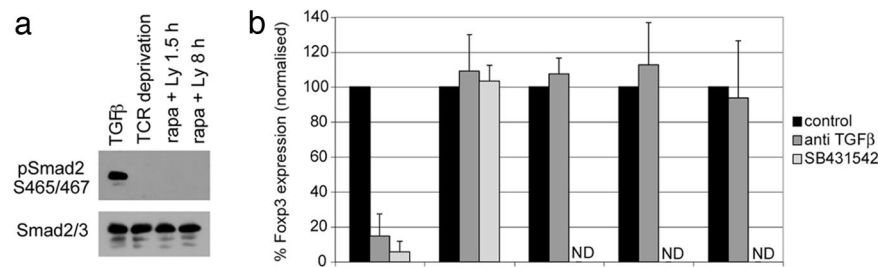
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**Fig. 4.** No apparent TGF $\beta$  involvement in Foxp3 induction by PI3K and mTOR inhibitors. (a) Naive LN CD4 T cells were activated for 18 h in serum-free AIM-V medium and then exposed to TGF $\beta$  (1 ng/ml, 90 min, lane 1); TCR signal deprivation (90 min, lane 2); or TCR signal deprivation, rapamycin, and LY294002 (90 min for lane 3, 8 h for lane 4). Blots were sequentially probed with anti-pSmad2 (S465/467) and anti-Smad2/3. (b) Naive LN CD4 T cells activated as in a were deprived of TCR signals and TGF $\beta$ , and PI3K/mTOR inhibitors were added as indicated. Cultures were supplemented with neutralizing anti TGF $\beta$  (3  $\mu$ g/ml) or the Smad kinase inhibitor SB431542. Foxp3 expression in the presence of anti TGF $\beta$  (dark gray bars) or SB431542 (light gray bars) was determined 2 days later and normalized to control cultures (black).

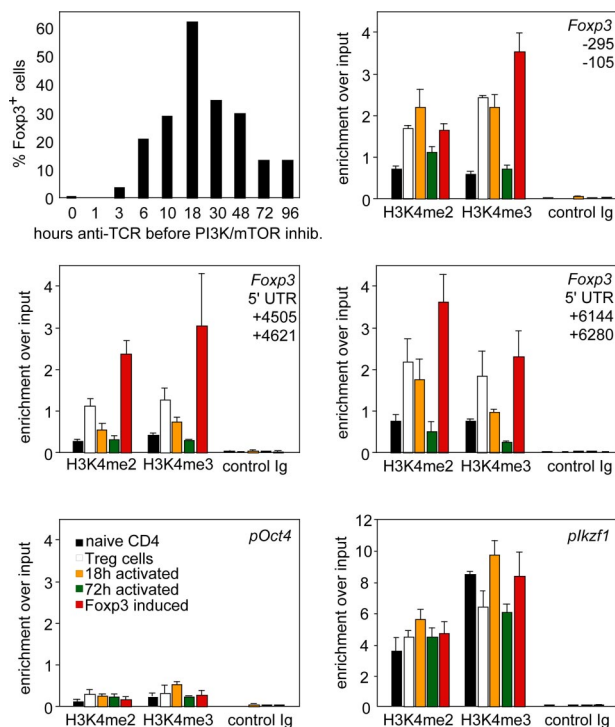
scripts were enriched for processes of cellular, macromolecular and primary metabolism, cell division, and cell cycle and for the functional terms nucleotide binding, electron transporter, and kinase regulatory activity. Down-regulated transcripts represented distinct processes, in particular transcriptional regulation. Only a minority of genes that were coregulated in *ex vivo* Treg cells and Foxp3 induced cells were known genomic targets of Foxp3 (Fig. S2). MicroRNAs are important mediators of posttranscriptional gene regulation and naive CD4 T cells and Treg cells express distinct microRNAs (31). Of the 10 microRNAs we profiled, 7 showed Treg-like expression in Foxp3-induced cells (Fig. 3c). Taken together, our analysis suggests that PI3K/mTOR signaling controls not only Foxp3 and its direct targets, but a wider Treg-like transcriptional program (30).

**No Detectable Involvement of TGF $\beta$  in Foxp3 Induction by PI3K and mTOR Inhibitors.** Because TGF $\beta$  is a powerful inducer of Foxp3 expression (14–17) and synergizes with PI3K/mTOR inhibitors (Fig. 1), we addressed its requirement in this system. TGF $\beta$  binding induces phosphorylation of receptor-associated Smad2 and Smad3, providing a sensitive indicator of TGF $\beta$  signaling. pSmad2 (S465/467) was readily detectable in cells exposed to TGF $\beta$  (Fig. 4a lane 1) but not in cells subjected to TCR signal deprivation (Fig. 4a, lane 2) or PI3K and mTOR inhibition (Fig. 4a, lanes 3 and 4). Neutralizing TGF $\beta$  antibodies and the Smad kinase inhibitor SB 431542 (32) blocked Foxp3 induction by TGF $\beta$ , but did not affect Foxp3 induction by PI3K/mTOR inhibitors (Fig. 4b). Hence, TGF $\beta$  appears dispensable for Foxp3 induction by TCR signal deprivation and PI3K/mTOR inhibition.

**Histone Modifications Mark a Window of Opportunity for Foxp3 Induction by PI3K and mTOR Inhibition.** T cell activation was required for Foxp3 induction, and Foxp3 inducibility was maximal in T cells activated for 18 h before PI3K/mTOR inhibition. Earlier addition of inhibitors blocked activation (ref. 19 and data not shown), and Foxp3 induction was inefficient at later time points (Fig. 5 Top Left). Hence, the competence for Foxp3 expression induced by activation of CD4 T cells is transient and continued TCR signaling antagonizes Foxp3 inducibility.

The expression of the *Foxp3* locus is intimately linked to its chromatin structure (33, 34). Permissive posttranslational histone modifications are found in Treg cells at the *Foxp3* promoter, the intronic differentially methylated region 3 (DMR3), and the recently described +2079 to +2198 enhancer (33–35). To explore how continued TCR signaling reduces the competence of CD4 T cells to express Foxp3, we considered that chromatin marks can provide important information not only about the actual expression, but also the potential for the expression of developmentally regulated loci (36). We used ChIP (chromatin immunoprecipitation) to analyze histone modifications at the *Foxp3* locus in male (XY) cells

(Foxp3 is X-linked). We compared CD4 cells activated for 18 h (high potential for Foxp3 induction, no Foxp3 expression) to the same cells after 72 h of TCR stimulation (reduced potential for Foxp3 induction, no Foxp3 expression) and CD4 cells activated for 18 h and then exposed to PI3K/mTOR inhibitors (high Foxp3 expression). *Oct4*, which is silent in T cells, and the actively transcribed *Ikzf1* (Ikaro) locus served as controls (Fig. 5). Interestingly, H3K4 di- and trimethylation was found near the *Foxp3* TSS (34) and the 5' UTR not only in Foxp3<sup>+</sup> cells but also in 18-h activated CD4 T cells, which had the potential for Foxp3 induction but did not actually express Foxp3. In contrast, H3K4me2 and -3 were lost after 72 h of continuous TCR signaling (Fig. 5). These data



**Fig. 5.** Inducibility of Foxp3 by PI3K and mTOR inhibition is transient, and H3K4 methylation at the Foxp3 TSS and 5' UTR marks the inducible state. Naive CD4 T cells were activated for the indicated time with anti-TCR and anti-CD28 and then cultured for 36 h with LY294002 and rapamycin (Upper Left). Naive CD4 T cells (black), Treg cells (white), naive CD4 T cells activated for 18 h (orange) or 72 h (green) or induced to express Foxp3 by PI3K/mTOR inhibitors (red) were examined for H3K4me2 and H3K4me3 by ChIP and qPCR near the Foxp3 TSS (–295 to –105) and within the 5' UTR (+4505 to +4621 and +6144 to +6280). Primer positions are indicated (34). *pOct4* and *plkzf1* are shown as controls.

link H3K4 methylation to the potential for Foxp3 expression. Conversely, the TCR signaling-induced loss of this permissive chromatin mark correlates with declining competence for Foxp3 expression.

## Discussion

We have shown that TCR signaling via PI3K p110 $\alpha$ , p110 $\delta$ , Akt, and mTOR controls Foxp3 expression in activated CD4 lineage thymocytes and peripheral T cells. Inhibition of this signaling network conferred *de novo* expression of Foxp3 and Treg-like mRNA and miRNA profiles. Conversely, constitutive activation of the PI3K/AKT/mTOR network in PTEN-deficient T cells reduced Foxp3 inducibility, which was restored by PI3K inhibition. Foxp3 induction by TCR signal deprivation and PI3K/mTOR inhibition shares features with the development of natural Treg cells. TGF $\beta$ , although synergistic, is not required for the induction of Foxp3 expression in the thymus (8, 9) or in response to TCR signal deprivation and PI3K and mTOR inhibition (this article), and both are markedly enhanced by costimulatory signals (refs. 2 and 3 and Fig. S3). Whereas CD28 signals can interfere with adaptive Treg differentiation driven by TGF $\beta$  (e.g., 13, 17), cooperation between TGF $\beta$  and CD28 was reported for the *Foxp3* enhancer element at +2079 to +2198 (35).

Evidence for an involvement of the PI3K/Akt/mTOR network in Treg differentiation and function has been accumulating: Treg cell numbers increase in the thymi of PI3K p110 $\delta$ -deficient mice (37), rapamycin can promote Treg cell differentiation in specific settings (18–20), and exciting data published while this manuscript was under review indicate that Akt signaling interferes with Foxp3 expression *in vitro* and *in vivo* (38). Our data provide a rationale for these genetic and pharmacological data by demonstrating that (i) TCR signaling controls Foxp3 expression via a signaling network with the key components PI3K $\alpha$  and  $\delta$ , Akt, and mTOR, the mammalian target of rapamycin, and (ii) the timing of PI3K/Akt/mTOR inhibition relative to TCR signaling is critical for the outcome. Interestingly, Akt signaling appears to be compromised in a PTEN-dependent fashion in established Treg cells (39).

Directing cell fate choice in the immune system by pharmacological means is potentially attractive, and a growing number of suitable compounds are approved for clinical use. The ability of small molecule inhibitors to block PI3K/mTOR/Akt signaling temporarily rather than permanently may be beneficial because constitutive p110δ deficiency promotes the differentiation of Treg cells in the thymus, but impairs their subsequent maintenance in the periphery (26, 37).

In our experiments, the duration of TCR signaling affects the probability of Foxp3 induction. Weak TCR signals and limited costimulation had been linked to Foxp3 induction (13, 17, 40). We find higher Foxp3 induction rates by optimal anti-TCR/CD28 signaling followed by TCR signal deprivation and PI3K/mTOR inhibition, consistent with a recent two-stage model of Treg differentiation in which TCR signals are required for the up-regulation of genes like *Cd25* that predispose to Treg differentiation, but not for the subsequent induction of Foxp3, which occurs in the absence of additional TCR signals (41). Treg development can result from the recognition of tissue-specific antigens expressed by thymic medullary epithelium (42). Such antigens are expressed in a mosaic fashion (43) and may therefore induce transient rather than sustained TCR signals, which in turn could contribute to the

outcome of Foxp3 induction versus negative selection. Our data further show that continued TCR signaling extinguishes the competence for Foxp3 induction in naive CD4 T cells. ChIP analysis identified H3K4me2 and -3 near the *Foxp3* TSS and within the 5' UTR as a mark for the inducibility of Foxp3, because these permissive histone modifications were induced by the activation of naive CD4 T cells and persisted upon the induction of Foxp3 expression, but declined in parallel with the loss of Foxp3 inducibility in response to continued TCR signaling. The challenge now is to understand how signaling, transcription factors, and chromatin components cooperate to translate the duration of TCR signaling into a determinant of Treg cell fate choice.

## Materials and Methods

**Cell Sorting and Culture.** Animal work was done under the Animals (Scientific Procedures) Act, U.K. Lymph node (LN) cells or thymocytes from wild-type (C57BL/6, BALB/c, or C57BL/6  $\times$  129) AND TCR transgenic (44) *Rag1*<sup>-/-</sup> or *IckCre* Pten<sup>lox/lox</sup> mice (29) were sorted by flow cytometry as described in ref. 31. LN CD4<sup>+</sup> CD25<sup>-</sup> CD62L<sup>hi</sup> T cells or CD4<sup>+</sup> CD8<sup>-</sup> CD25<sup>-</sup> thymocytes were activated with plate bound anti-TCR $\beta$  (H57; 200 ng/ml; PharMingen) and anti-CD28 (2  $\mu$ g/ml, PharMingen). AND TCR transgenic cells were also activated with B10.BR antigen presenting cells and pigeon cytochrome-C peptide 81–104 (44). Intracellular staining for Foxp3 protein was done as advised by the manufacturers (eBiosciences). Anti-pS6 Ser-235/236 (Cell Signaling; catalog no. 2211) was used with the eBioscience Foxp3 staining kit and anti rabbit IgG-FITC or IgG-Cy5 (Jackson ImmunoResearch).

**RT-PCR, Northern Blot Analysis, and Immunoblotting.** Total RNA was isolated by using RNA-Bee (Tel-Test) and reverse transcribed. Real-time PCR analysis was normalized to the geometric mean of *Ywhaz* and *Ube2L3* as described in ref. 31. The following primer sequences were used: *Ywhaz*, CGTTGTAGGAGCCCGTAGGTCAT (forward), TCTGTTTGCAGCATTGGG (reverse); *Ube2L3*, AGGAGGCTGATGAAGGAGCTTGA (forward); TGGTTTGAATGGATACTCTGCTGGA (reverse); and *Foxp3*, ACTCGATGTTCGCTACTTCTAG (forward); GGCGGATGGCATTCTCCAGGT (reverse). Immunoblotting was performed as described in ref. 31. Quantitative real-time RT-PCR of miRNAs used gene specific RT primers and TaqMan MicroRNA Assay Mix (Applied Biosystems) as described in ref. 31.

**Chromatin Immunoprecipitation.** ChIP was done as described in ref. 45, using 20  $\mu$ g of chromatin mixed with 80  $\mu$ g of *Drosophila* S2 chromatin with 2.5  $\mu$ l of anti-H3K4me2 (Upstate; catalog no. 07-030), anti-H3K4me3 (Abcam; catalog no. ab8580-100), or rabbit anti mouse IgG (Dako Cytomation; catalog no. Z0259). The following primer sequences (5' to 3'): *Foxp3* -295 to -105 (TSS; numbering according to ref. 34) CATTGATACCTTTTACCTCTGTGGTG (forward), GTGTGTGCTGATAATTCAGGGT (reverse); *Foxp3*, 5' UTR +4505 to +4621 (DMR3) GTTGTGACAACAGGGCCCCAG (forward), CACTGTCTGTTGGGGCGTCT (reverse); *Foxp3*, 5' UTR +6144 to +6280 (*exon-1*) CAACCTCTCTGACTCTGCCTTCA (forward), GGAACGTGCTAGTGGGAAGTGTACT (reverse); *pOtc4* GTGAGCCGTCTTCCACCAGG (forward), GGGTGAGAAGGC-GAAGTCTGAA (reverse); and *plkzf1* CCAGTTTCAAGGACTCGGT (forward), TCGGGGAACCGGGACAC (reverse).

**Gene Expression Arrays and Bioinformatics Analysis.** Affymetrix mouse genome 430 2.0 arrays were used in duplicate. Analysis was done in Bioconductor, using robust multi array normalization ([www.bioconductor.org](http://www.bioconductor.org)). Individual *P* values were corrected according to Benjamini and Hochberg (46). Only transcripts with both an adjusted *P* value <0.05 and a fold-change of >1.5 were considered differentially expressed. Functional annotation used DAVID (<http://david.abcc.ncifcrf.gov>).

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